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ORIGINAL ARTICLE

Transcriptome analysis of *Lactobacillus rhamnosus* GG strain treated with prebiotic - bovine lactoferrin under a cold environment

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Abstract

Lactoferrin (LF) is secreted by mammals and displays extensive biological effects. We previously reported that bovine LF (BLF) can boost the cold tolerance of a well-applied probiotic strain, *Lactobacillus rhamnosus* GG (LGG), to grow robustly under a cold environment, but the molecular mechanism is not clear. Here, RNA-seq analysis was conducted to ascertain molecular pathways underlying cold tolerance exerted by BLF. LGG was cultured in a cold environment (22 °C) in the presence or absence of BLF. Transcriptome analysis indicated that BLF significantly elicited 1.2−3.2 fold (log2 Fold change) higher expression levels of genes related to stress, defense, cell division, and transporter in LGG, including the genes CspA, LytR, XRE, MerR, and GpsB. The KEGG pathway and GO analyses confirmed that BLF can modulate a few central pathways to boost the growth of LGG. BLF also reduced metabolic pathways involved in purine, amino acid, pyrimidine, one-carbon metabolism, and secondary metabolites in LGG. We speculate that the reduction of the above pathways may play key roles to reduce energy requirement and maintain carbon metabolism balance in LGG for surviving and growing in a cold state, and BLF can be an excellent prebiotic to LGG cultured in this cold condition (22 °C). Overall, this study uncovers the molecular effects of BLF on LGG.

Keywords: Lactoferrin, *Lactobacillus rhamnosus* GG, Transcriptome

1. Introduction

Lactoferrin (LF) is an 80-kDa iron-binding protein present mostly in the milk and exocrine fluids of mammals, and it has been shown to display extensive biological effects including anti-inflammatory, antimicrobial, and immune-regulation properties. Thus, the application of LF to various fields has been studied [1−3]. For example, supplementation with LF or specific probiotic strains has been suggested to be a good strategy for controlling several bacterial and fungal vaginal infections, and a simultaneous combination of LF preparations and specific lactobacilli strains can improve women's health through probiotic and prebiotic input [4]. Although some studies have attempted to combine the health-promoting effects of LF use and specific probiotic bacterial strains together, LF may exert either growth inhibitory or promotion effects on specific probiotic strains [5−7]. Notably, LF exerts more favorable growth inhibitory effects on most pathogens than on probiotic strains, and the inhibitory effect of bovine LF (BLF) on selected probiotic bacteria is at least 4-fold lower than that on several pathogenic bacteria [6,8]. In particular, we also revealed that at 22 °C, the growth of *Bifidobacterium breve*,

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Lactobacillus coryniformis, *Lactobacillus delbrueckii*, *Lactobacillus acidophilus*, *Bifidobacterium angulatum*, *Bifidobacterium catenulatum*, and *Lactobacillus paraplantarum* were completely blocked. However, these probiotics started regrowth in the presence of BLF (1–32 mg/mL) in a significant and dose-dependent manner, supporting that LF possesses prebiotic activity on specific probiotic strains [7]. In other words, BLF supplementation can boost the stress tolerance responses of specific probiotic strains. However, the molecular mechanism of LF in boosting stress tolerance of specific probiotic strains is still unclear.

RNA sequencing (RNA-seq) and transcriptome analysis are good approaches for transcriptome profiling whereby deep-sequencing technologies are employed to directly determine the cDNA sequence. Moreover, this approach has a relatively low background compared with the use of microarrays. Most importantly, it provides the absolute expression value of each gene, thereby making it possible to evaluate the expression abundance of each gene in the same life stage [9,10]. Furthermore, RNA-seq can be applied to identify the transcriptional profiles of specific bacteria, and even strains or species whose genome have not yet been sequenced [9]. As for lactic acid bacteria, genes associated with the utilization of tetrasaccharides by *Lactobacillus ruminis* L5 were identified using transcriptome techniques [11], and the growth phase-associated changes in the transcriptome profiles of *Lactobacillus rhamnosus* GG in industrial-type whey medium was also evaluated by RNA-seq [12]. Finally, RNA-seq has been conducted on *Lactobacillus acetotolerans* F28 growing in 12% ethanol to determine important genetic mechanisms for the short and long term adaption to ethanol stress [13]. Collectively, transcriptome analysis and RNA-seq can provide detailed information regarding global changes in gene expression and molecular pathways under particular conditions.

L. rhamnosus GG strain (ATCC 53103; LGG) was originally isolated from the human gastrointestinal tract as a probiotic bacterial strain [14,15], and it has been shown to exert a variety of biological activities including antibacterial activity against a variety of bacterial species [16,17], immune modulation activities [18–20], and clinical trials also point out that this strain may reduce adiposity, body weight, and weight gain in human, and these suggest that LGG can be applied in the treatment of obesity [21]. Furthermore, several reports, including studies in different clinical conditions, human volunteer studies, and epidemiologic surveillance, all support that the LGG strain is safe for human consumption even in large amounts [22,23]. Collectively, LGG is currently one of the most widely studied, well-documented, and safe probiotic bacterial strains; in our recent report, we demonstrated that BLF can strongly promote the growth rate of LGG and enhance the cold tolerance of LGG as well [6,7]. However, the mechanism of BLF becoming a prebiotic agent is still not well understood. In the current study, we employ transcriptome analysis to study the molecular mechanism of how BLF can strongly boost the growth of LGG grown under a cold environment. We identified evidence that BLF supplement can modulate a few central metabolic pathways in LGG and it also modulates specific stress-, defense-, cell cycle-, amino acid synthesis- and transporter-related genes to encourage LGG adapt to the cold environment. This study investigates the role of BLF on the putative molecular regulatory network of LGG, and the identified differentially expressed genes (DEGs) in Kyoto Encyclopedia of Genes and Genomes (KEGG) term-enriched terms may also help to secure core candidate genes for further functional studies on the molecular mechanism of stress tolerance in LGG. In the large scale fermentation of LGG that the combination of BLF and LGG could decrease the heat energy for LGG culture but also increase the output of the probiotics fermentation industry.

2. Materials and methods

2.1. Bacterial strains, media, and growth conditions

LGG ATCC 53103 was purchased from the Food Industry Research and Development Institute, Taiwan. Bacteria were first activated and maintained in MRS medium at 37 °C under anaerobic conditions for 48 h. To evaluate the molecular mechanisms of BLF on the growth of LGG, a spectrophotometric turbidity bioassay was performed as described previously [7]. The broth of activated LGG (optical density was about 1.2) was diluted and adjusted to 5×10^6 cfu/mL and was further cultured at 22 °C in MRS broth medium supplemented with different concentrations of BLF when needed. Briefly, BLF was serially diluted in MRS broth, and 90 μL of the prepared BLF was transferred into a 96-well culture plate containing 90 μL of the prepared probiotic solution to achieve final LF concentrations of 32, 16, 8, 4, 2, and 1 mg/mL. For the control cultures (0 mg/mL of LF), MRS was added instead of the BLF solution. The growth responses of LGG at 22 °C in the presence or

absence of BLF were measured by determining the optical density at 595 nm at different time intervals, and results are expressed as means ± SD. Moreover, at different time intervals, about 2 mL of bacterial cultures were harvested from 96-well plates and were further centrifuged at 6000×*g* for 10 min at 4 °C to collect the bacterial cells. Finally, the harvested bacterial cells were stored at −80 °C and subjected to further RNA-seq analysis.

2.2. RNA isolation and RNA-seq

Total RNAs from four samples were extracted using TRIzol reagent (Invitrogen) and QIAGEN RNeasy mini kit. The total concentration, RIN, 23S/16S, and size of the RNA samples were determined using Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Kit). The purity of the samples was evaluated by NanoDrop™. The total RNA was used to construct a cDNA library, and for transcriptome library construction, several workflows were completed, including rRNA depletion, RNA fragmentation, first strand cDNA synthesis, second strand cDNA synthesis, ends repair & A-tailing, adaptor ligation, and PCR amplification. For example, DNase I digestion was used to degrade double-stranded and single-stranded DNA present in RNA samples. Then, total RNA samples were treated with Ribo-Zero™ Magnetic Kit to deplete rRNA, and RNA molecules were fragmented into small pieces using fragmentation reagent. As for cDNA synthesis, the first-strand cDNA was generated using random primers reverse transcription, and was followed by second-strand cDNA synthesis. Next, the synthesized cDNA was subjected to end-repair and then was 3′ adenylated. Finally, adapters were ligated to the ends of these 3' adenylated cDNA fragments.

As for PCR amplification, the cDNA fragments were amplified with adapters from the previous step, and PCR products were purified with the XP beads, and then dissolved in EB (RB solution). Next, the libraries were assessed for quality and quantity using two methods: first, the distribution of the fragment sizes was checked by Agilent 2100 bioanalyzer, and the library was quantified using real-time quantitative PCR (qPCR) (TaqMan Probe). Finally, qualified libraries were processed on cBot to generate the cluster on the flowcell, and the amplified flowcell was pair end sequenced on the Illumina System. Initially, according to the growth curve of LGG treated with or without the addition of BLF, we focused on analyzing the transcriptome profiles of LGG co-cultured with 1 mg/mL BLF at 60 h and 72 h time intervals.

2.3. Transcriptomics analysis

A series of quality control steps were conducted on the raw reads obtained by sequencing. For example, we removed reads containing adaptor sequence, reads with an unknown base number greater than 5% of the total sequence length, reads with Q $\leq$ 15, and reads where the base number accounts for more than 20% of the total read base number of the reads. Then, the clean reads for each sample were aligned to the reference genome using HISAT software (v2.0.1-beta) [24].

DEGs were identified as described previously using PossionDis software [25], and the log2 (fold change) $\geq$ 1 and false discovery rate $\leq$ 0.005 were set as the significant threshold values to S using the KEGG pathway database [26], and Q value $\leq$ 0.05 was set to ascertain the significantly enriched pathways. Moreover, GO enrichment analysis was conducted to determine the main molecular functions, cellular components, and biological processes associated with DEGs by consulting the to GO database (http://www.geneontology.org), and the corrected *p*-value ≤0.05 was set to ascertain significantly enrichment GO terms. The cluster analysis for genome-wide expression with open source clustering software was determined as described previously [27,28].

3. Results

3.1. Influence of BLF on the growth of LGG

To investigate the transcriptome profile of LGG in the presence or absence of BLF addition, LGG was cultured under a cold environment with a series of BLF concentrations (from 0 to 32 mg/mL). As expected, in the absence of BLF treatment (Fig. 1), the growth of LGG was strongly retarded by lower temperature incubation up until 60 h. LGG started slow regrowth at 72 h, whereby the optical density (OD) of LGG elevated from below 0.2 (60 h) to about 0.5 (72 h) in 600 nm. The regrowth of LGG at 72 h may be due to the adaption of bacteria to sub-optimal growth conditions. In contrast, in the presence of BLF (1−32 mg/mL), LGG started regrowth in a significant and dose-dependent manner at 48 h, 60 h, and 72 h time points. Most importantly, the growth of LGG treated with either concentrations of BLF was observed to be at least 2-fold higher than the control at 60 h and 72 h time intervals. Notably, 1 mg/mL BLF was enough to display strong prebiotic activity towards LGG especially at 60 h and 72 h time points when compared to the control (0 mg/mL). Thus, the transcriptome profiles of LGG

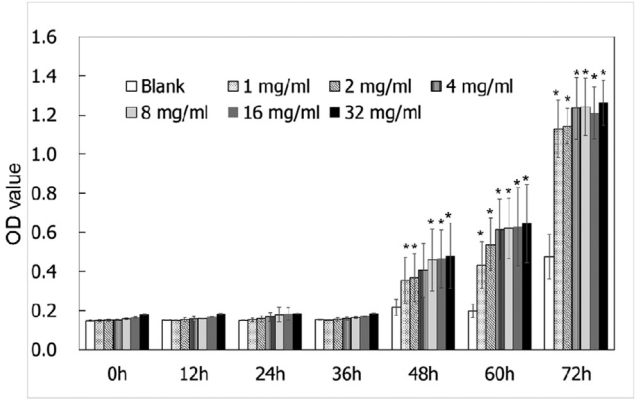

*Fig. 1. Effects of bovine lactoferrin (BLF) on the growth of probiotics. Lactobacillus rhamnose GG strain (LGG) was cultured in MRS medium with and without various concentrations of BLF at 22 °C. The growth responses of LGG were measured by determining the optical density. *Significant differences in the probiotic growth with and without BLF ($p < 0.05$).*

treated with 1 mg/mL BLF at 60 h and 72 h were subsequently investigated. For comparison, the transcriptome profiles of LGG in the absence of BLF at 60 h and 72 h time points were also determined.

3.2. The transcriptome of LGG in the presence or absence of BLF

In Table 1, sequence statistics of LGG are shown. We detected a total of 7.9 million pairs of reads for the control LGG at 60 h, 8.0 million for the BLF-treated LGG at 60 h, 7.0 million for the control LGG at 72 h, and 7.3 million for the BLF-treated LGG at 72 h. Moreover, saturation curves and gene coverage indicated a completely adequate sequencing depth (Fig. S1 and S2). When compared to the known mRNA, the gene expression numbers in each sampler or the mean RPKM (Reads Per Kilo bases per Million reads) was 2358 in the control LGG at 60 h, 2371 in the BLF-treated LGG at 60 h, 2312 in the control LGG at 72 h, and 2304 in the BLF-treated LGG at 72 h (Fig. S3). Moreover, high and low gene expressions are usually defined by an arbitrary threshold, and here, we defined genes with RPKM smaller than 10 as low expression genes, genes with RPKM levels between 10 and 100 as intermediate expression, and genes with RPKM levels above 100 as high. In Fig. S4, the boxplots of gene expression numbers of each sample indicate that the four samples showed similar gene expression levels. Furthermore, the hierarchical clustering of gene expression (Fig. S5) indicate that BLF treatment affects the gene expression pattern, and the most differentially expressed genes between control and BLF-treated LGG had relatively low to medium expression levels. These up-regulated and down-regulated DEGs were further analyzed using PossionDis software as described below.

Table 1. RNA-sequence statistics of LGG. LGG were cultured at 22 °C in the presence or absence of BLF incubation from 0 to 72 h. Control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analyzed using transcriptome analysis at 60 h and 72 h time interval.

	Control (60 h)	BLF treated (60 h)	Control (72 h)	BLF treated (72 h)
Total CleanReads	7,926,940	8,047,484	7,048,228	7,338,506
Total Mapped Reads (%)	58.87	62.23	64.82	65.31
Perfect Match (%)	21.13	21.31	24.31	24.81
Unique Match (%)	57.64	61.44	64.52	64.97

3.3. Identification of DEGs

To identify genes involved in prebiotic effects of BLF, we used PossionDis software to determine the DEGs between the samples. At the 60 h time point (Fig. S6), transcriptome analysis identified a total of 115 DEGs in BLF-treated LGG, including 59 up-regulated genes and 56 down-regulated genes, and a total of 2293 genes were significantly non-regulated. At the 72 h time interval (Fig. S6), transcriptome analysis revealed that only 1 gene was up-regulated and 6 genes were down-regulated in BLF-treated LGG, and a total of 2327 genes were significantly non-regulated. In addition, we further extracted and summarized some DEGs that are useful for illustrating the molecular mechanism of BLF on promoting LGG as indicated below.

3.4. Stress and defense response in LGG treated with BLF supplementation

As described above, a total of 115 DEGs were identified in BLF-treated LGG at 60 h using PossionDis analysis. In the current study, LGG were cultured in a cold environment (22 °C), and thus, we speculate that the expression of genes involved in cell division or stress responses would be modulated by BLF supplementation. Indeed, among the 115 DEGs, expression of specific genes involved in stress or defense response pathway were promoted by BLF supplementation (Table 2). Here, about ten genes involved in stress, defense, or cell division responses were increased by 1.0–3.2 fold (log2 Fold change) in BLF-treated LGG, and the data of *p*-value and False discovery rate (FDR, *q*-value) have been shown as well. The FDR values for above genes all are below 0.004 (between 0.004 to smaller than 0.00001), and this supports that these genes are differently expressed in a considerately significant manner. For example, expression of ABC transporter permease (MFS

Table 2. The fold change value of genes, grouped into stress, defense and cell division in LGG treated with and without BLF incubation at 60 h time interval. LGG were cultured at 22 °C in the presence or absence of BLF incubation from 0 to 72 h. Gene expression levels of control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analyzed using RNA-seq analysis at 60 h and 72 h time interval. A total of 59 genes were up-regulated in BLF-treated LGG at 60 h time interval, and genes which are related to stress, defense and cell division genes are further summarized here. The p-value and FDR (False discovery rate, q-values) < 0.05 indicate these genes are differently expressed in a statistically significant manner.

Gene ID	Log2 Fold Change (treated/control)	Protein encoded	Functional group	FDR	p-value
LRHM_RS11665	3.22	ABC transporter permease	Stress response	0.00035	0.00009
LRHM_RS02895	2.20	Cold-shock protein (beta-ribbon, CspA family)	Defense mechanism	0.00000	0.00000
LRHM_RS12300	2.08	PTS galactitol transporter subunit IIA	Defense mechanism	0.00000	0.00000
LRHM_RS10720	1.77	LytR family transcriptional regulator	Defense mechanism	0.00000	0.00000
LRHM_RS02995	1.49	XRE family transcriptional regulator	Defense mechanism	0.00100	0.00028
LRHM_RS11355	1.33	XRE family transcriptional regulator	Defense mechanism	0.00049	0.00013
LRHM_RS02560	1.18	MerR family transcriptional regulator	Stress response	0.00133	0.00038
LRHM_RS13960	1.14	Peptide ABC transporter ATP-binding protein	Stress response	0.00468	0.00154
LRHM_RS07940	1.06	Peptide ABC transporter ATPase	Stress response	0.00000	0.00000
LRHM_RS10305	1.01	Glycine/betaine ABC transporter ATP-binding protein	Stress response	0.00007	0.00002
LRHM_RS07080	1.21	Cell division protein	Cell division and cell cycle	0.00000	0.00000

transporter) and Cold-shock protein (CspA family) were enhanced by 3.2 and 2.2 fold, respectively. Moreover, genes encoding LytR and MerR family transcriptional regulator were up-regulated by 1.77 fold and 1.18 fold, respectively. Finally, a gene encoding MerR family transcriptional regulator (activator of the bmr gene), which can promote transcription of various stress regulons, was also up-regulated by 1.18 fold. Collectively, these findings indicated that BLF supplementation could protect LGG against cold stress by up-regulating a series of stress- and defense-related genes especially at 60 h. Notably, a cell division protein, GpsB, was also up-regulated by 1.21 fold (log2 Fold change). In contrast, at 72 h, there were only seven DEGs that were modulated by BLF supplementation, of which there were no stress- or defense-related genes that were up-regulated by BLF supplementation.

3.5. Metabolic pathways and gene ontology (GO) analysis of DEGs

KEGG pathway and GO analyses were conducted to interpret the potential roles played by the DEGs that promote LGG growth exerted by BLF. At 60 h (Fig. 2A), the identified significantly DEGs were mapped into four categories of pathways: cellular processes (3 genes), environmental information processing (7 genes), genetic information processing (7 genes), and metabolism (53 genes). In contrast, at 72 h (Fig. 2B), the identified significantly DEGs were mapped to only the metabolism category (8 genes). Collectively, there were more categories of pathways that were modulated by BLF at 60 h but not at 72 h.

In addition, the top 20 enriched KEGG pathways are further shown in Fig. 3. Here, at 60 h (Fig. 3A), genes involved in "alanine, aspartate and glutamate metabolism", "one carbon pool by folate", "metabolic pathways", "biosynthesis of secondary metabolites", "biosynthesis of antibiotics", "purine metabolism", and "pyrimidine metabolism" categories were the most significantly enriched. On the other hand, at 72 h (Fig. 3B), genes involved in "alanine, aspartate and glutamate metabolism", "one carbon pool by folate", "metabolic pathways", "biosynthesis of secondary metabolites", "biosynthesis of antibiotics", and "purine metabolism" categories were the most significantly enriched. Therefore, the above findings from samples collected at 60 h and 72 h confirmed that BLF exerted predominantly on similar (metabolic pathways) as well as specific pathways to promote the growth of LGG.

We further compared the DEGs that were down-regulated or up-regulated by BLF supplementation. At the 60 h time point (Fig. 4A), the most enriched pathways in BLF-treated contained down-regulated DEGs. For example, some genes involved in purine metabolism, alanine, aspartate, and glutamate metabolism, pyrimidine metabolism, biosynthesis of secondary metabolites, one carbon pool by folate, and biosynthesis of antibiotics were identified to be down-regulated by BLF supplementation. Moreover, genes of some pathways were also up-regulated, including RNA degradation, nicotinate and nicotinamide metabolism, butanoate metabolism, cell cycle-Caulobacter, base excision repair, DNA replication, peptidoglycan biosynthesis, aminoacyl-tRNA biosynthesis, cysteine and methionine

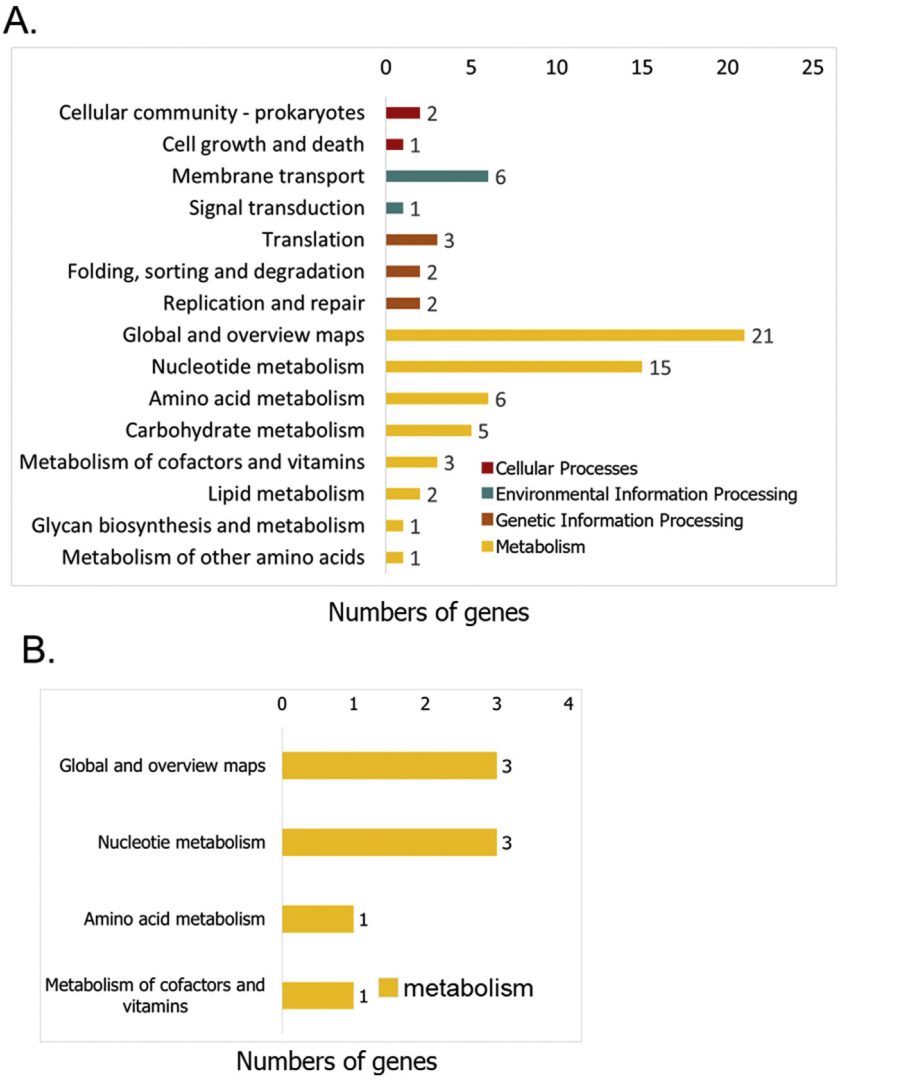

Fig. 2. ***KEGG classification of lactoferrin treated VS non-treated LGG strain analyzed by using PossionDis method.*** *LGG were cultured at 22 °C in the presence or absence of BLF incubation from 0 to 72 h. Control (0 mg/mL) and treated (1 mg/mL) bacterial samples were analyzed at 60 h (A) and 72 h (B) time interval. The horizontal axis represents the number of genes in that pathway, and vertical axis represents the category of pathway.*

metabolism, and galactose metabolism. These up-regulated genes of pathways involved in DNA replication, cell wall synthesis, and cell cycle could explain some roles of BLF in promoting the growth of LGG. In contrast, at 72 h (Fig. 4B), the most enriched pathways in BLF-treated LGG were only down-regulated DEGs of pathways including purine metabolism, biosynthesis of antibiotics, biosynthesis of secondary metabolites, metabolic pathways, one carbon pool by folate, and alanine, aspartate, and glutamate metabolism, and no up-regulated DEGs of pathways were identified in LGG at 72 h.

In Fig. 5, GO functional annotation histograms of the DEGs in BLF-treated and control samples are shown. Here, a total of 376 DEGs were clustered into 24 functional groups for the samples harvested at 60 h (Fig. 5A). Moreover, these DEGs were distributed into 10, 7, and 7 subcategories of biological process, cellular component, and molecular function categories, respectively. Furthermore, the largest subcategories within biological process were "cellular process", "metabolic process", and "membrane part", "cell", and "cell part"; Within the molecular function category, DEGs were distributed within "catalytic activity", and "binding". Moreover, for samples collected at 72 h (Fig. 5B), only 18 DEGs were clustered into 8 functional groups. These DEGs were distributed into 2, 4, and 2 subcategories of biological process, cellular component, and molecular function categories, respectively. Furthermore, the "cellular process", "metabolic process",

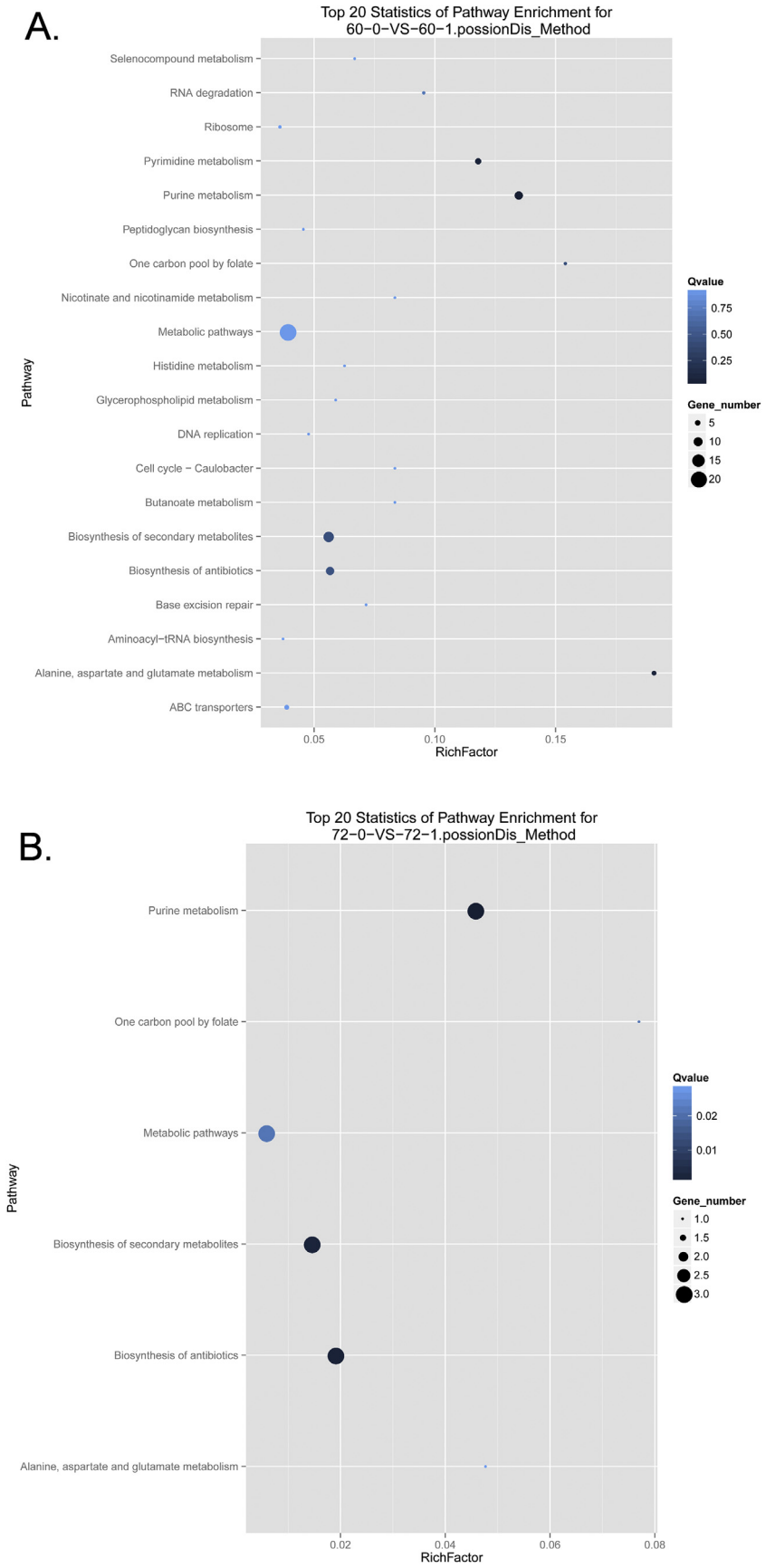

Fig. 3. ***Scatterplot of the top 20 enriched KEGG pathways of lactoferrin treated VS non-treated LGG.*** *LGG were cultured at 22 °C in the presence or absence of BLF incubation from 0 to 72 h. Control (0 mg/mL) and treated (1 mg/mL of BLF) bacterial samples were analyzed at 60 h (A) and 72 h (B) time interval. The pathway names are represented on the vertical axis, and the horizontal axis represents the pathways corresponding to the rich factor. The ratio of the number of DEGs and all annotated genes in the pathway is defined as the rich factor, and $q \leq 0.05$ as significantly enriched.*

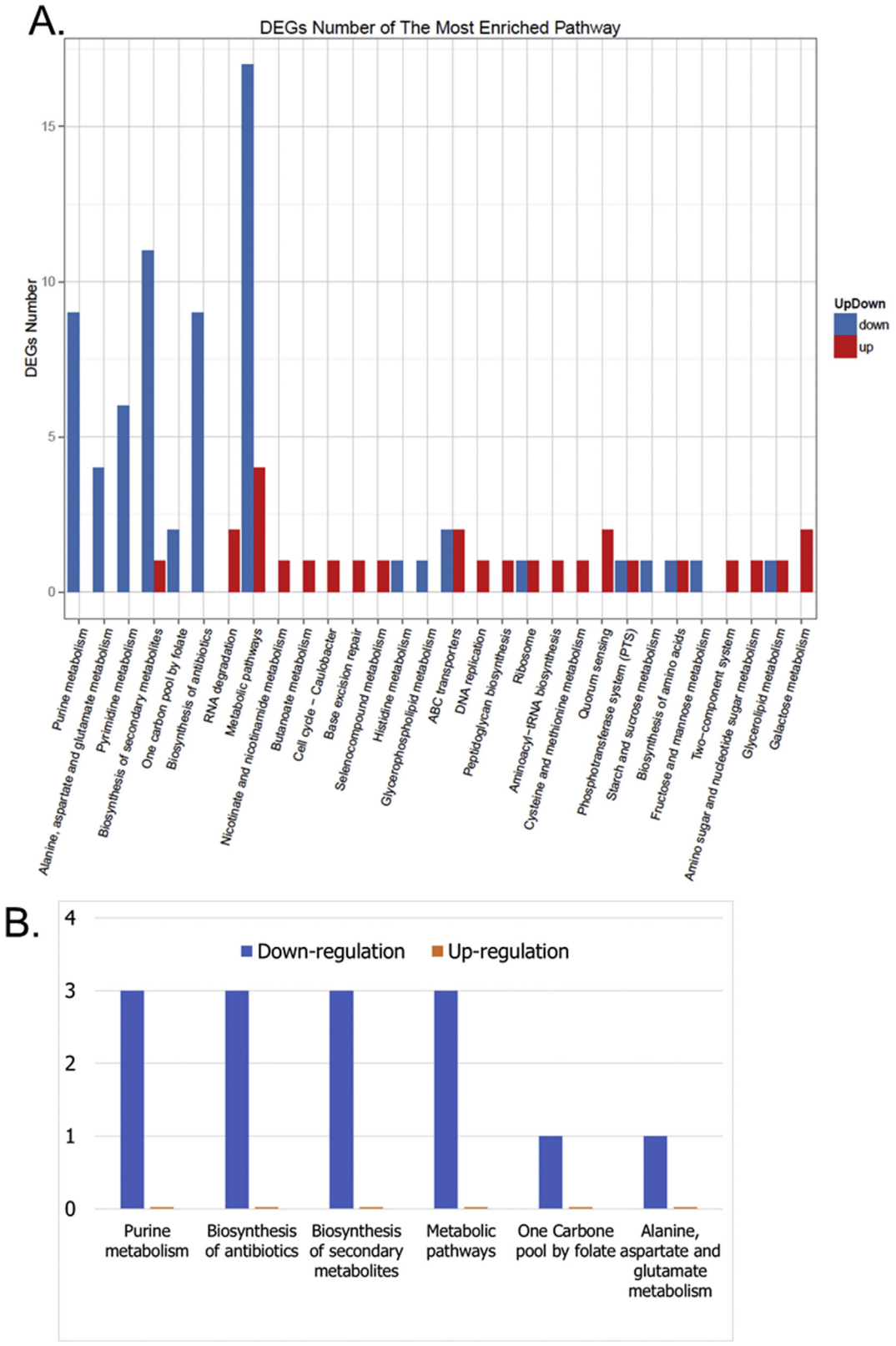

Fig. 4. ***DEGs numbers of the most enriched pathway of lactoferrin treated VS non-treated LGG strain.*** *LGG were cultured with or without 1–32 mg/mL BLF incubation from 0 to 72 h. Control (0 mg/mL) and treated (1 mg/mL) bacterial samples were analyzed at 60 h (A) and 72 h (B) time interval. The horizontal axis represents the number of genes enriched in that pathway, and these genes of pathways were shown according to the levels of the enriched factors as the strongest enriched DEGs of particular pathway were shown at the left side.*

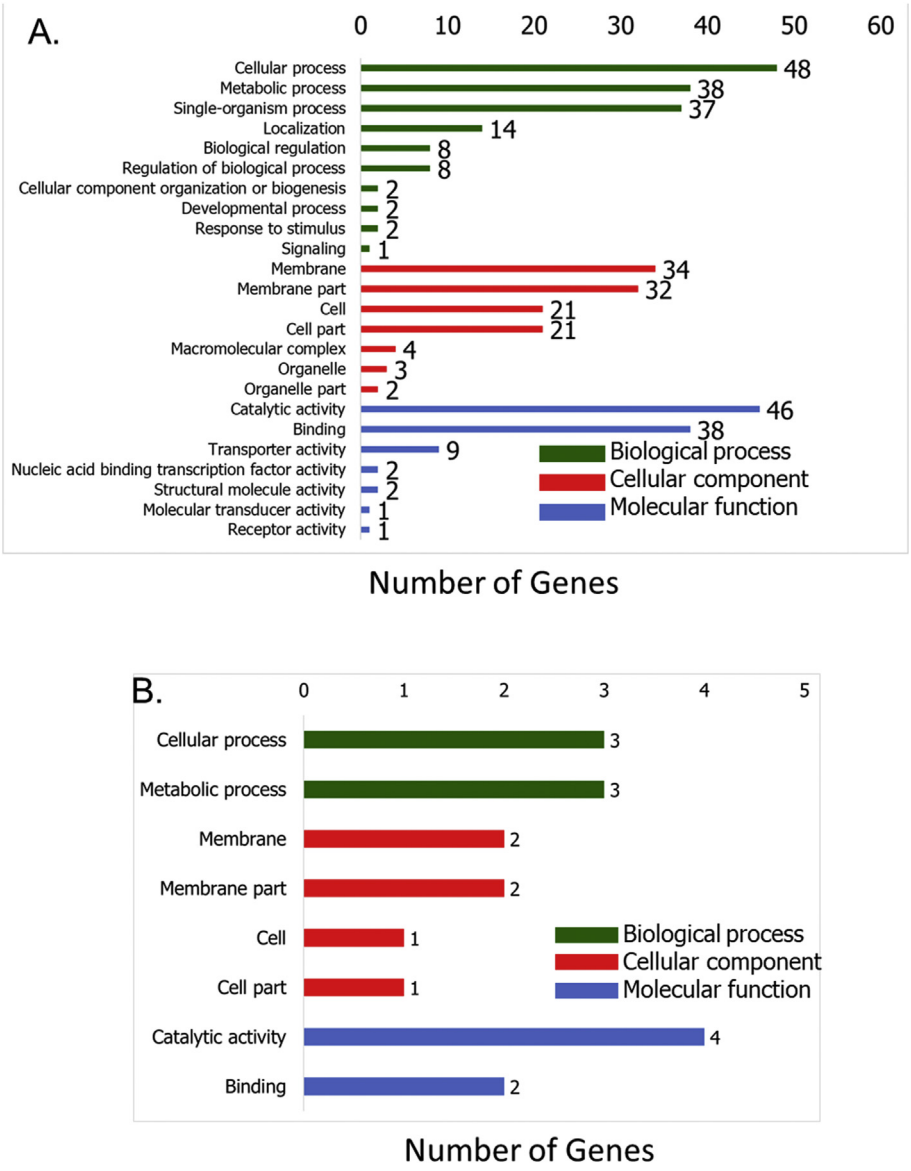

Fig. 5. ***Functional analysis of GO annotation.*** *The horizontal axis represents the gene number, and the number of genes is considered the difference in the proportion of the total, and the vertical axis represents the three basic GO categories. LGG were cultured with or without 1–32 mg/mL BLF incubation from 0 to 72 h. Control (0 mg/mL) and treated (1 mg/mL) bacterial samples were analyzed at 60 h (A) and 72 h (B) time interval.*

"membrane part", "cell" and "cell part", "catalytic activity", and "binding" subcategories were the most distributed functions.

4. Discussion

In our recent reports, BLF supplementation was shown to promote the growth of a series of specific probiotic strains in a dose-dependent manner, as well as promote and re-start the growth of LGG following retardation by a cold stress environment (about 22 °C) [6,7]. However, to date, the molecular mechanism of BLF as a prebiotic agent is still unclear. Therefore, in the present study, a global transcriptomic analysis between BLF-treated and untreated LGG cultured at 22 °C was conducted. Here, we investigated the global gene expression profile at two time points, 60 h and 72 h. Firstly, at 60 h, the growth of LGG control was still strongly retarded by sub-optimal culturing conditions (cold environment) but BLF supplementation had already boosted the growth of LGG by this time in a dose-dependent manner. Second, at 72 h, LGG seemed to have adapted to the cold stress and started to regrow again. However, BLF supplementation still strongly enhanced the growth of LGG at this period

of time. Notably, the lowest concentration of BLF supplementation tested (1 mg/mL) was enough to promote the growth of LGG both at 60 h and 72 h. Therefore, we investigated the transcriptome profiles of LGG treated with 1 mg/mL BLF between two time intervals. Our data reveals that BLF indeed exerts similar but also different global gene expression profiles in LGG samples harvested at the two time points.

As shown in the present study, the significantly up-regulated core genes in BLF-treated LGG at 60 h can be classified into the stress- or defense-related responses. For example, expression of genes related to an MFS transporter, a cold-shock protein (CspA family), and a gene encoding LytR family transcriptional regulator and MerR family transcriptional regulator (activator of bmr gene) are strongly enhanced by BLF supplementation. The roles of these genes in prokaryotic bacteria have been previously reported to help different bacterial species to survive or grow under sub-optimal conditions. For example, the MFS transporter has been annotated as a member of the family of multidrug resistance transporters that can act as a defense mechanism against inhibitory compounds by extruding a wide variety of structurally dissimilar substrates from the cytoplasm including antibiotics, bile salts, and peptides [29,30]. In addition, this transporter also displays physiological roles in allowing the bacterium to survive in its ecological niche [31]. In the present study, we also identified expression of another two ABC transporter-related genes that are promoted by BLF. Thus, BLF supplementation may promote several transporter-related genes to allow for LGG growth in a cold environment.

As for the BLF-upregulated Csps that we identified, previous findings have suggested that Csps may regulate the expression of cold-induced genes, such as anti-terminators [32], and the expression of CspA in a *Lactobacillus casei* strain was induced after a temperature downshift from 37 °C to 20 °C. In addition, a CspA mutant strain was constructed and displayed reduced growth rate compared with the wild type at both optimal and low temperatures, revealing that CspA plays an important role in the physiology of *L. casei* related to cell growth, carbohydrate transport, carbon metabolism, and cold shock [33]. Therefore, CspA may play similar roles in LGG, and the up-regulated CspA could play major roles in driving LGG growth in the cold environment.

Regarding the BLF-upregulated LytR related gene (LytR family transcriptional regulator), the LytR-Cps-Psr family proteins are commonly present in Gram-positive bacteria, and these proteins are reported to be required for the surface attachment of both capsular polysaccharides and teichoic acids of pneumococci. Furthermore, the ΔlytR mutant can impair the growth of *Streptococcus pneumoniae*, and addition of recombinant LytR protein to the culture medium restores the growth in a dose-dependent manner [34], indicating that LytR also plays physiology roles in maintaining the growth of specific bacterial strains. In support of this, several studies have identified links between growth phase, cell density, and expression of the LytR/CpsA/Psr family in several bacterial strains [35,36]. Therefore, in the present study, the enhanced expression of LytR family transcriptional regulator by BLF may boost the function of an integral component of the membrane to help LGG grow in a cold environment.

In this study, BLF also enhanced the expression of *bmrR*, which is a general stress protein that belongs to a group of transcriptional activators (MerR family) of the bmr-bmrR operon. This regulator controls the expression of the Bmr efflux pump in response to a diverse array of cationic antibiotics, dyes, and disinfectants, which are also transported by Bmr and other efflux pumps [37]. Therefore, the roles of MerR family could be similar to the roles of MFS transporters and ABC transporters that are responsible for extruding unfavorable components of bacteria to survive in stress conditions.

At 60 h, BLF supplementation was also upregulated the expression of a gene encoding the cell division protein GpsB by 1.21 fold (Log2 Fold change). It has been shown that GpsB is widely conserved in the Firmicutes phylum and is linked to cell growth and division in Gram-positive bacteria [38–40].

Collectively, previous studies have found that some cold stress-, defense-, and cell division-related genes or products are involved in survival and growth of specific prokaryotic bacterial species. Our data provide new evidence supporting that LGG may also employ these mechanisms to survive in a cold environment, and BLF supplementation could promote these protection effects.

We have explained the roles of BLF on up-regulation of expression of some core genes that can enhance the growth of LGG under a cold environment at 60 h. In support of this, the KEGG pathway and the GO functional analyses have confirmed that BLF supplementation modulated robust genes of pathways that are involved to metabolism, DNA replication, and cell wall synthesis (Fig. 4). However, at 72 h, BLF supplementation seems to have affected another mechanism to boost the growth of LGG. It should be noted that, at 72 h, the LGG control had already adapted to the cold environment and started

to partially regrow again (Fig. 1). Thus, we believe that some genes or products involved in cold stress responses must have been revoked by LGG control at this stage. Indeed, at this time point, our findings from the PossionDis, KEGG pathway, and GO analyses all support that BLF plays fewer regulating roles on transcriptome gene profiles than that at 60 h time point. For example, at 72 h, the PossionDis analysis revealed only one gene that was up-regulated and six genes that were down-regulated in BLF-treated LGG. Moreover, for KEGG pathway analysis of the 60 h time interval, the significantly DEGs were mapped into four categories of pathways: cellular processes (3 genes), environmental information processing (7 genes), genetic information processing (7 genes), and metabolism (53 genes).

In contrast, at 72 h, the significantly DEGs were mapped to only one category, the metabolism category (8 genes). In concordance with the fewer pathways modulated by BLF at 72 h using KEGG pathway analysis, the GO term analysis also revealed that fewer pathways and functions were modulated by BLF supplementation at 72 h. Furthermore, PossionDis approach analysis identified that only seven DEGs were modulated by BLF and no stress- or cell division-related DEGs were affected by BLF incubation. Because LGG control were able to adapt to the cold stress state and restarted to grow again at 72 h, we speculate that the stress-, defense-, or cell division-related genes were revoked in the absence of BLF supplementation at 72 h. Thus, the transcriptome analysis identified fewer molecular pathways and genes that were further modulated in BLF-treated LGG. However, BLF strongly promoted the growth of LGG at both time points studied. Therefore, these DEGs and pathways that were identified to be modulated by BLF at both time points may play core roles to help LGG resist the cold environment.

Our data show that, in BLF-treated LGG at 60 h, although quite a few genes involved in pathways related to metabolism, DNA replication, and cell wall synthesis were promoted by BLF treatment, the most enriched KEGG pathways in BLF-treated LGG were actually down-regulated pathways. These down-regulated pathways include some DEGs related to purine metabolism, alanine, aspartate, and glutamate metabolism, pyrimidine metabolism, biosynthesis of secondary metabolites, one carbon pool by folate, and biosynthesis of antibiotics. Furthermore, in BLF-treated LGG, although fewer numbers of DEGs and pathways were identified and mapped at 72 h, the most enriched KEGG pathways were still down-regulated pathways. These include genes related to purine metabolism, biosynthesis of antibiotics, biosynthesis of secondary metabolites, metabolic pathways, one carbon pool by folate, and alanine, aspartate, and glutamate metabolism. Therefore, when the growth of LGG control cells were still blocked by the cold environment (at 60 h) or when the LGG had adapted to the cold environment and started to regrow again (at 72 h), BLF supplementation contributed to down-regulation of the above nucleotide-, specific amino acid-, and carbon pool-related pathways. Overall, we believe these down-regulated metabolic pathways, which are involved in purine, amino acid, pyrimidine, one-carbon metabolism, and secondary metabolites may reduce energy requirements and maintain carbon metabolism balance to help BLF-treated LGG to survive and grow in cold stress conditions. To support our findings, previous transcriptome analyses in *L. acetotolerans* F28, *Lactobacillus plantarum*, and beer-spoilage *L. acetotolerans* have suggested that reducing "amino acid transport and metabolism" pathways might be a necessary strategy to maintain carbon and nitrogen metabolism balance to survive under ethanol stress or viable putative non-culturable state [13,41,42].

In conclusion, LGG is one of the most widely investigated and well-documented probiotic bacterial strains, and it has been applied to various fields. LF is a natural protein of mammals that displays extensive physiological functions. We demonstrated previously that LGG could resist the antibacterial activities of BLF, and BLF supplementation could further boost the cold tolerance of LGG. However, it was still unclear about the molecular mechanism of BLF on promoting the growth of LGG. In the present study, though transcriptome analysis, BLF supplementation has been shown to modulate a number of genes that are involved in many central metabolic pathways. In Fig. 6, the main molecular roles of BLF in promoting the growth of LGG are summarized according to the findings from current study. BLF supplementation could elevate higher expression levels of specific stress, defense, cell division, and transporter response genes that have been reported to be relevant to growth and survival in several bacterial species. In addition, BLF supplementation could also reduce a series of metabolic pathways involved in purine, amino acid, pyrimidine, one-carbon metabolism, and secondary metabolites, and these may help LGG to reduce energy requirements and maintain carbon metabolism balance in order to survive and grow in a cold environment. The combination of the above effects and molecular pathways may render the ability of BLF to strongly boost the cold tolerance of LGG.

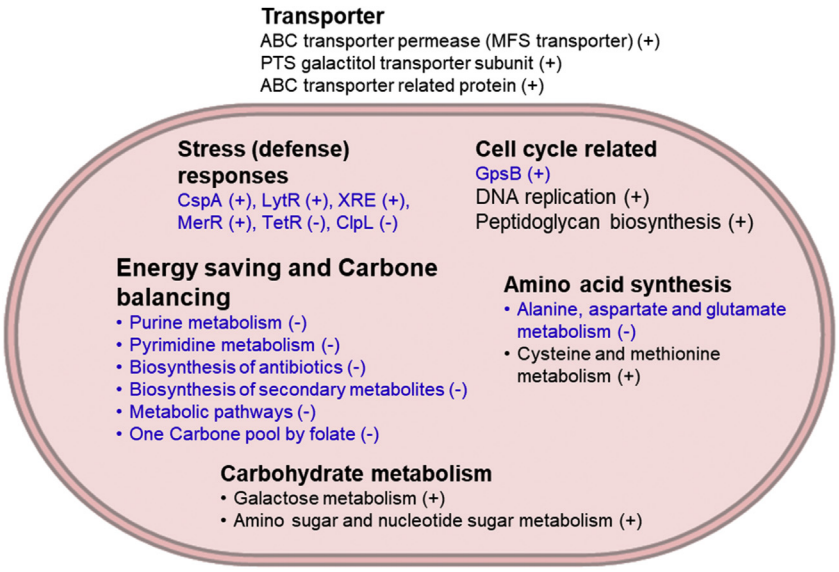

Fig. 6. The central molecular pathways or genes which are modulated by BLF supplementation in LGG under a cold environment incubation. Increased and decreased expression is indicated by plus symbol '+' and minus symbol '-' respectively.

However, further experiments are needed to confirm the adaptive behavior of LGG exerted by BLF by using different experiment design or approaches, and to elucidate the adaptive behavior of LGG exerted by BLF could help to both elucidate the prebiotic activity of BLF and understand the cold tolerance mechanism of LGG. For example, the identified DEGs in KEGG term-enriched terms may help to secure core candidate genes for further functional studies on the molecular mechanisms of stress tolerance in LGG. BLF could be potentially applied in future to promote or maintain the growth of specific probiotics cultures under sub-optimal conditions. However, as described elsewhere in this study, the prebiotic ability of BLF is actually bacterial strain-dependent, and thus, BLF may modulate different DEGs and putative molecular regulatory networks in different probiotic strains.

Conflict of interest

No conflict of interest exists.

Acknowledgements

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Appendix A. Supplementary material

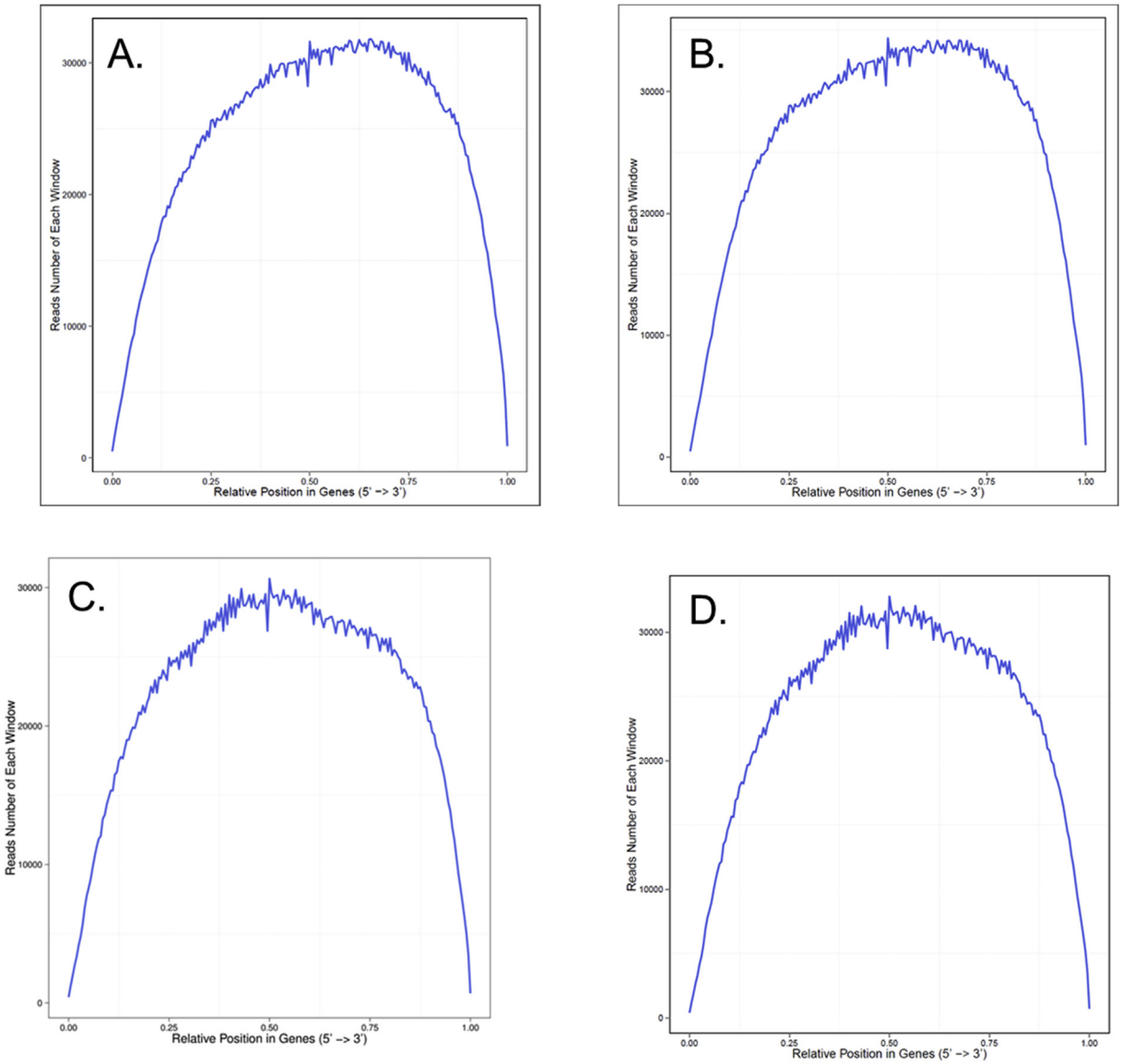

Fig. 1S. Reads distribute on references gene. LGG were cultured at 22 °C with or without BLF incubation from 0 to 72 h. Gene expression levels of control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analyzed using RNA-seq analysis at 60 h and 72 h time interval. (A) samples of 60 h (control); (B) samples of 60 h (BLF treated one); (C) samples of 72 h (control); (D) samples of 72 h BLF treated one.

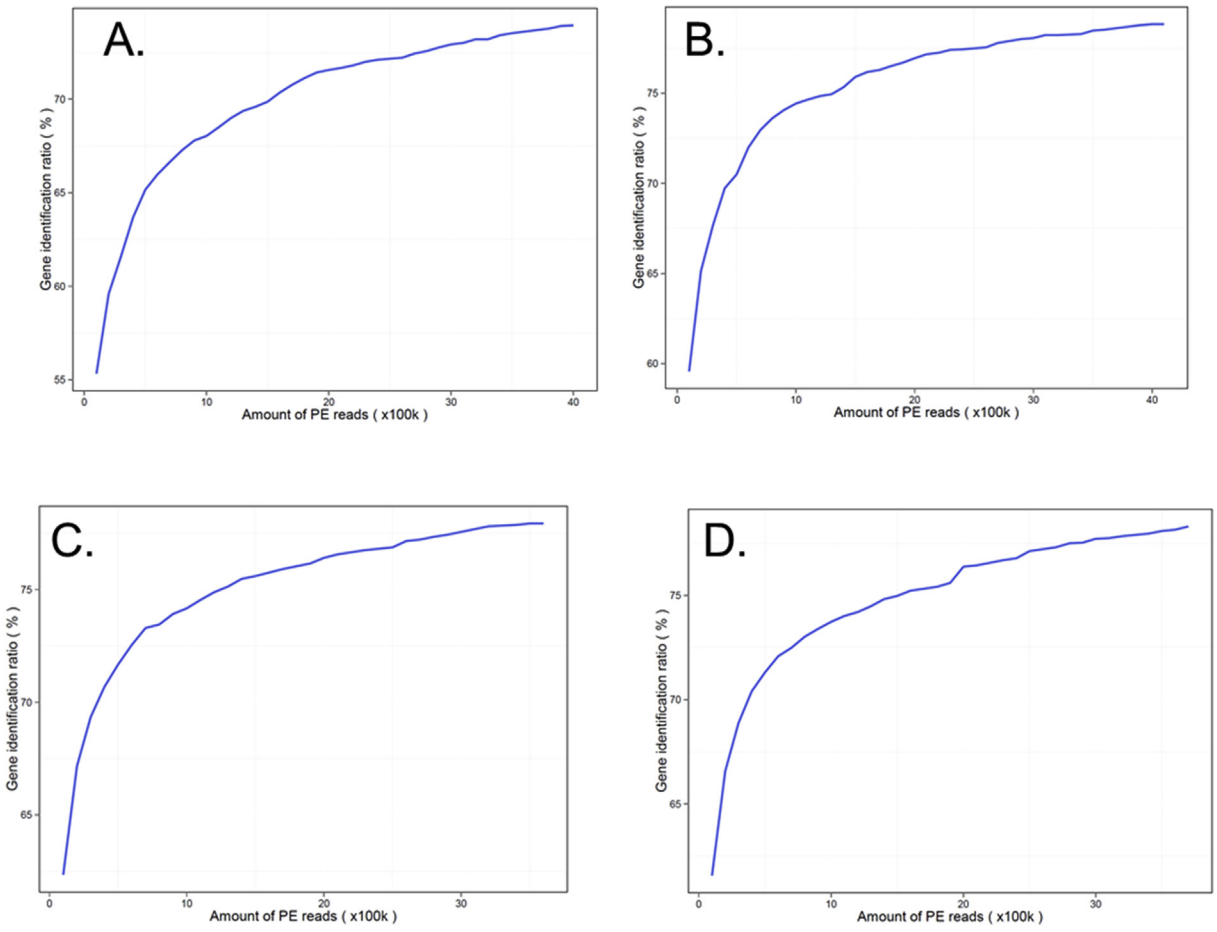

Fig. 2S. Curve of sequencing saturation .X-axis shows the number of clean reads, units is 100 k-extreme value is currently the volume of sequencing. Y-axis shows the ratio of identified gene number to number of total gene reported in database. (A) samples of 60 h (control); (B) samples of 60 h (BLF treated one); (C) samples of 72 h (control); (D) samples of 72 h BLF treated one.

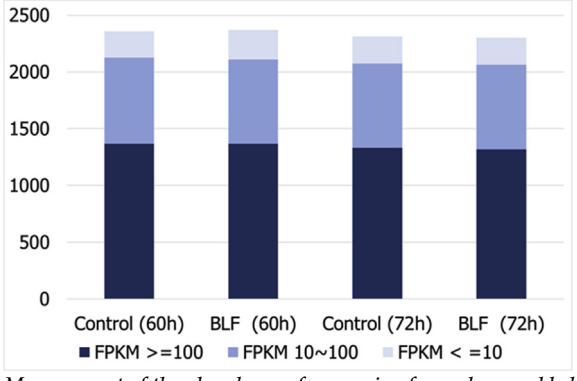

Fig. 3S. Gene expression in each sample. Measurement of the abundance of expression for each assembled transcript was done using the Fragments per Kilobase of exon model per Million mapped reads (FPKM) values.

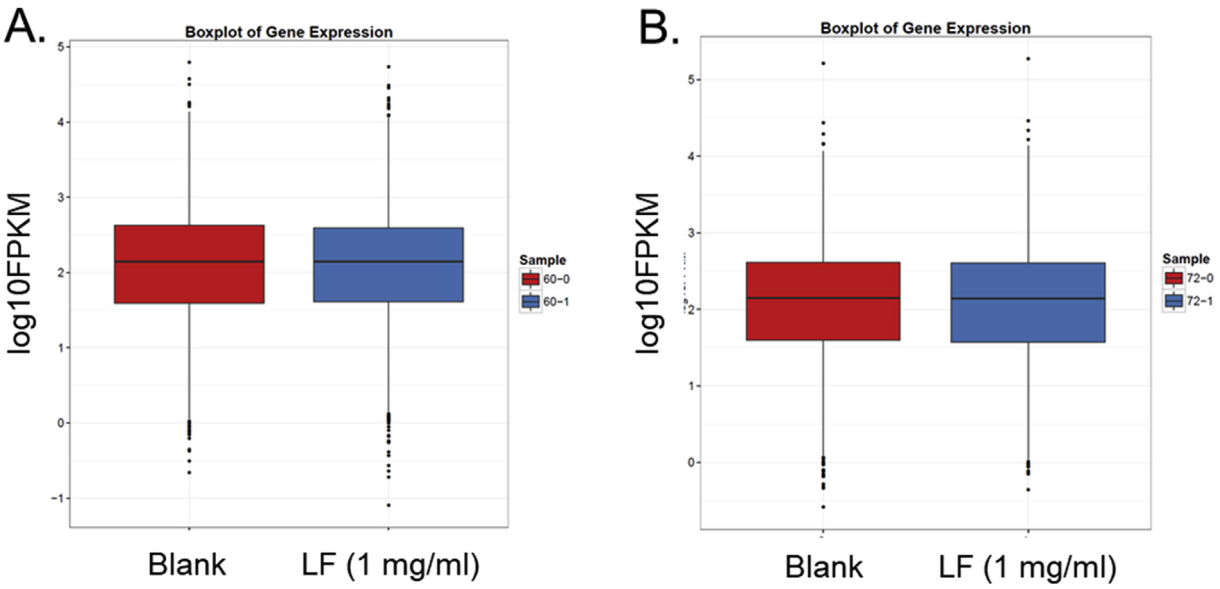

Fig. 4S. Boxplot of gene expression. Gene expression in each samples. LGG were cultured at 22 °C with or without BLF incubation from 0 to 72 h. Gene expression levels of control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analyzed using RNA-seq analysis at 60 h (A) and 72 h (B) time interval. Measurement of the abundance of expression for each assembled transcript was done using the Fragments per Kilobase of exon model per Million mapped reads (FPKM) values. The box depicts the interquartile range between the first and third quartiles (25 th and 75 th percentiles, respectively) and the line inside the box indicates the median.

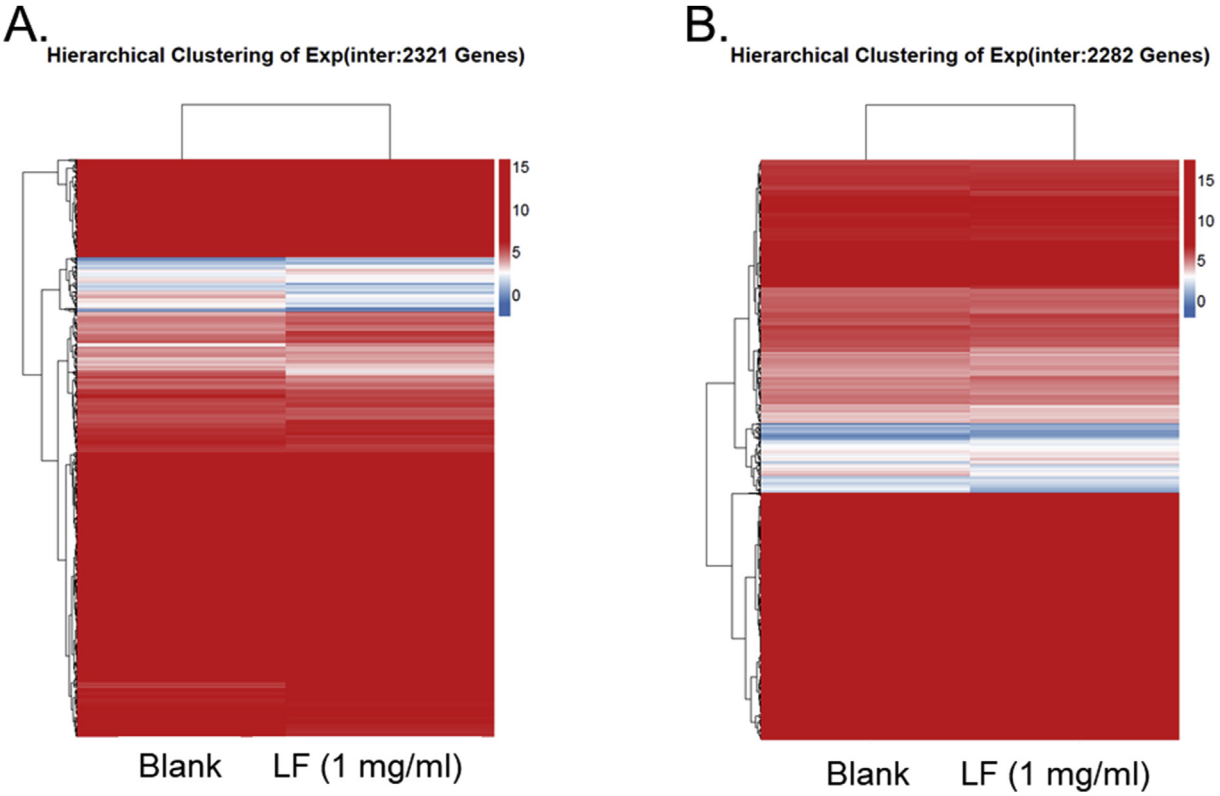

Fig. 5S. Hierarchical clustering of interactively expression genes LGG were cultured at 22 °C in the presence of absence of BLF incubation from 0 to 72 h. Gene expression levels of control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analysed using RNA-seq analysis at 60 h (A) 72 h (B) time interval.

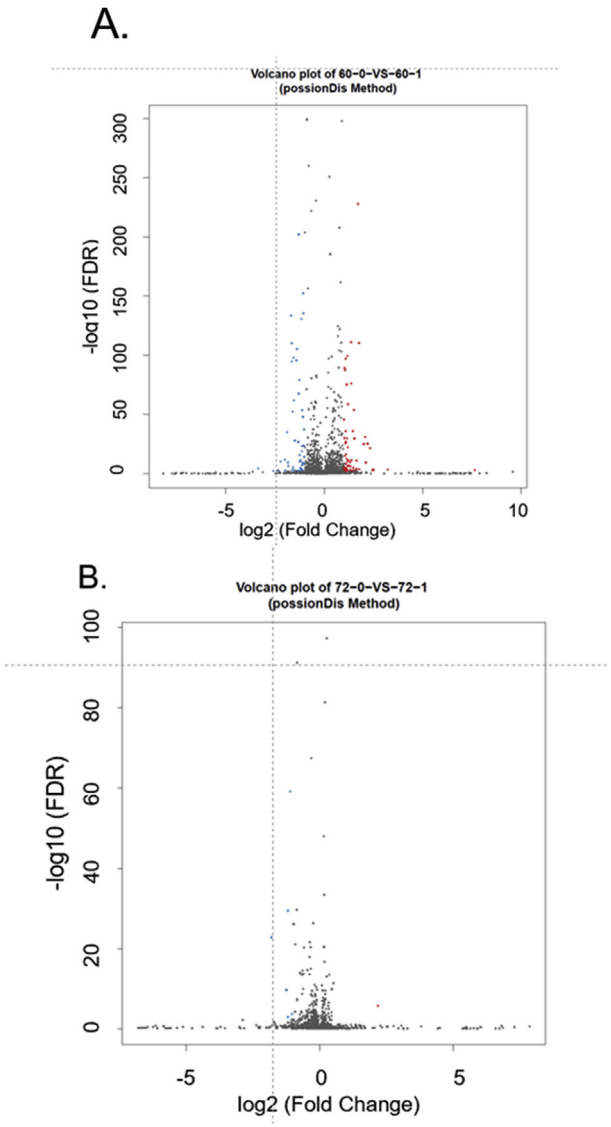

Fig. 6S. Volcano plot of different expression genes (DEGs) between samples treated with or without BLF analyzed using possionDis method. LGG were cultured at 22 °C with or without BLF incubation from 0 to 72 h. Gene expression levels of control (0 mg/mL) and BLF-treated (1 mg/mL) bacterial samples were analyzed using RNA-seq analysis at 60 h (A) and 72 h (B) time interval; Up-regulated significantly DEGs were shown in red (Log2FoldChange ≥1 or FDR ≤ 0.005), down-regulated significantly DEGs were shown in blue (Log2FoldChange ≤-1 or FDR ≤0.005), and non-regulated significantly DEGs were shown in grey (Log2FoldChange <1 or FDR >0.005).

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